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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

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TITLE OF THE INVENTION (500 characters max)

HCV REPLICONS CONTAINING NS5B FROM GENOTYPE 2B

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Respectfully submitted,

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EXPRESS MAIL CERTIFICATE

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TITLE OF THE INVENTION

HCV REPLICONS CONTAINING NS5B FROM GENOTYPE 2B

BACKGROUND OF THE INVENTION

The references cited in the present application are not admitted to be prior art to the claimed invention.

It is estimated that about 3% of the world's population are infected with the Hepatitis C virus (HCV). (Wasley *et al.*, 2000. *Semin. Liver Dis. 20*, 1-16.) Exposure to HCV results in an overt acute disease in a small percentage of cases, while in most instances the virus establishes a chronic infection causing liver inflammation and slowly progresses into liver failure and cirrhosis. (Iwarson, 1994. *FEMS Microbiol. Rev. 14*, 201-204.) Epidemiological surveys indicate HCV plays an important role in hepatocellular carcinoma pathogenesis. (Kew, 1994. *FEMS Microbiol. Rev. 14*, 211-220, Alter, 1995. *Blood 85*, 1681-1695.)

The HCV genome consists of a single strand RNA about 9.5 kb in length, encoding a precursor polyprotein about 3000 amino acids. (Choo *et al.*, 1989. *Science 244*, 362-364, Choo *et al.*, 1989. *Science 244*, 359-362, Takamizawa *et al.*, 1991. *J. Virol. 65*, 1105-1113.) The HCV polyprotein contains the viral proteins in the order: C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B.

Individual viral proteins are produced by proteolysis of the HCV polyprotein. Host cell proteases release the putative structural proteins C, E1, E2, and p7, and create the N-terminus of NS2 at amino acid 810. (Mizushima *et al.*, 1994. *J. Virol. 68*, 2731-2734, Hijikata *et al.*, 1993. *Proc. Natl. Acad. Sci. USA 90*, 10773-10777.)

The non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B presumably form the virus replication machinery and are released from the polyprotein. A zinc-dependent protease associated with NS2 and the N-terminus of NS3 is responsible for cleavage between NS2 and NS3. (Grakoui *et al.*, 1993. *J. Virol. 67*, 1385-1395, Hijikata *et al.*, 1993. *Proc. Natl. Acad. Sci. USA 90*, 10773-10777.)

A distinct serine protease located in the N-terminal domain of NS3 is responsible for proteolytic cleavages at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions. (Barthenschlager *et al.*, 1993. *J. Virol. 67*, 3835-3844, Grakoui *et al.*, 1993. *Proc. Natl. Acad. Sci. USA 90*, 10583-10587, Tomei *et al.*, 1993. *J. Virol. 67*, 4017-4026.) RNA stimulated NTPase and helicase activities are located in the C-terminal domain of NS3.

NS4A provides a cofactor for NS3 protease activity. (Failla *et al.*, *J. Virol.* 1994. *68*, 3753-3760, De Francesco *et al.*, U.S. Patent No. 5,739,002.)

NS5A is a highly phosphorylated protein conferring interferon resistance. (De Francesco *et al.*, U.S. Patent No. 6,383,768, Pawlotsky 1999. *J. Viral Hepat. Suppl. 1*, 47-48.)

NS5B provides an RNA-dependent RNA polymerase. (De Francesco *et al.*, International Publication Number WO 96/37619, published November 28, 1996, Behrens *et al.*, 1996. *EMBO 15*, 12-22, Lohmann *et al.*, 1998. *Virology 249*, 108-118.)

Efficient replication in cell culture has been associated with adaptive mutations that dramatically increase the frequency with which replication is established. (Ikeda *et al.*, 2002. *J. Virol. 76*, 2997-3006, Blight *et al.*, 2000. *Science 290*, 1972-1974, Lohman *et al.*, 2001. *J. Virol. 75,* 1437-1449, Kriege *et al.*, 2001. *J. Virol. 75,* 4614-4624.) Adaptive mutations in the HCV-con1 isolate have been localized to various non-structural genes, though substitutions upstream of the interferon sensitivity determining region in NS5A, for example S232I, appear to be the most effective. (Blight *et al.*, 2000. *Science 290*,1972-1974.) A 4 amino acid insertion in NS5A that is not commonly observed *in vivo* is important for replication in cell culture of the HCV-N isolate. (Ikeda *et al.*, 2002. *J. Virol. 76*, 2997-3006.) Substitutions in residue 470 combined with an NS5A-S232I adaptive mutation were found to be important for conferring cell culture replication to otherwise inactive replicons, including replicons derived from genotype 1b HCV-BK and genotype 1b HCV-H77. (Grobler *et al.*, 2003, *J. of Biological Chemistry 278*:16741-16746, 2003.)

SUMMARY OF THE INVENTION

The present invention features methods for enhancing the ability of a genotype 2b NS5B sequence to function in a replicon, for producing replicons containing a functional genotype 2b NS5B, and for using replicons to measure the ability of a compound to affect HCV replication that is sustained with the genotype 2b RNA polymerase. The ability to produce replicons containing a functional genotype 2b NS5B is illustrated by the production of chimeric replicons based on HCV genotype 1b where substantially all the NS5B sequence is replaced with a genotype 2b NS5B.

A HCV replicon is an RNA molecule able to autonomously replicate in a cultured cell, such as Huh7, and produce detectable levels of one or more HCV proteins. The HCV replicon expresses the HCV derived components of the replication machinery and contains cis-elements required for replication in a cultured cell.

Thus, a first aspect of the present invention features a method of enhancing the ability of a genotype 2b NS5B sequence to function in a replicon. The method comprises the step of altering the genotype 2b NS5B sequence to encode one or more adaptive mutations selected from the group consisting of: serine corresponding to position 24 of SEQ ID NO: 1;

isoleucine corresponding to position 31 of SEQ ID NO: 1; and leucine corresponding to position 392 of SEQ ID NO: 1.

SEQ ID NO: 1 provides a genotype 2b NS5B sequence. The exact amino acid numbering may vary for different replicon constructs. A "corresponding" position in different constructs can be identified by aligning the relevant regions in the constructs to achieve the greatest degree of homology around the position in question.

Another aspect of the present invention features a method of producing a chimeric replicon having a detectable level of expression. The method comprises the step of replacing substantially all of a NS5B sequence of a HCV replicon comprising a NS3-NS4-NS5A-NS5B ("NS3-5B") genotype 1b sequence with a genotype 2b NS5B encoding nucleic acid sequence.

Replacing "substantially all" indicates that all or most of a NS5B sequence is replaced and the resulting sequence is a NS5B genotype 2b sequence. Replacement can be achieved by different techniques such as recombinant nucleic acid techniques used to modify a nucleic acid sequence and/or synthesis techniques used to produce a particular sequence.

Another aspect of the present invention describes a chimeric replicon having a detectable level of expression. The chimeric replicon comprises an NS3-NS4-NS5A ("NS3-5A" sequence of a genotype 1b replicon and a genotype 2b NS5B encoding nucleic acid sequence.

Another aspect of the present invention features a recombinant cell. The cell comprises a chimeric replicon containing a NS5B genotype 2b sequence. The replicon replicates in the cell and replicon encoded protein are expressed.

Another aspect of the present invention features a method of measuring the ability of a compound to inhibit replicon activity. The method involves the steps of:

a) providing the compound to a recombinant cell containing a chimeric replicon with a NS5B genotype 2b sequence, and

b) measuring the ability of the compound to affect replicon activity.

Unless particular terms are mutually exclusive, reference to "or" indicates either or both possibilities. Occasionally phrases such as "and/or" are used to highlight either or both possibilities.

Reference to "comprises" is open-ended allowing for additional elements or steps. Occasionally phrases such as "one or more" are used with or without "comprises" to highlight the possibility of additional elements or steps.

Unless explicitly stated reference to terms such as "a" or "an" is not limited to one. For example, "a cell" does not exclude "cells". Occasionally phrases such as one or more are used to highlight the presence of a plurality.

Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples. The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates an amino acid sequence (SEQ ID NO: 1) showing different changes to a NS5B genotype 2b sequence. Z^1 is threonine or serine, where threonine was found in a genotype 1b NS5B, and serine was found in a genotype 2b. X^1 is asparagine or serine, where serine was identified as an adaptive mutation. X^2 is methionine or isoleucine, where isoleucine was identified as an adaptive mutation. X^3 is isoleucine or leucine, where leucine was identified as an adaptive mutation. The adaptive mutations N24S, M31I, and I392L individually conferred replication competence in chimera replicon constructs. The underlined sequence represents sequence derived from genotype 1b NS5B.

Figure 2 illustrates a nucleotide sequence (SEQ ID NO: 2) encoding the amino acid sequence of SEQ ID NO: 1. The underlined sequence represents sequence derived from a genotype 1b NS5B. X^1 is A or G, where G creates a serine adaptive mutation at amino acid 24. X^2 is G or T, where T creates an isoleucine adaptive mutation at amino acid 31. X^3 is A or C, where C creates a leucine adaptive mutation at amino acid 392. Y^1 is A found in a genotype 1b NS5B, where T was found in genotype 2b NS5B. Y^2 is C found in genotype 1b, where A was found in a genotype 2b NS5B. Y^3 is A found in a genotype 1b NS5B, where T was found in a genotype 2b NS5B. Y^4 is A found in a genotype 1b NS5B, where C was found in a genotype 2b NS5B. Y^5 is A found in a genotype 1b NS5B, where G was found in a genotype 2b NS5B. Y^6 is C found in a genotype 1b NS5B, where G was found in a genotype 2b NS5B. Y^7 is T found in a genotype 1b NS5B, where C was found in a genotype 2b NS5B. Y^8 is G found in a genotype 1b NS5B, where C was found in a genotype 2b NS5B. Y^9 is C found in a genotype 1b, where A was found in a genotype 2b NS5B.

Figure 3 illustrates a genotype 1b NS3-5A amino acid sequence (SEQ ID NO: 3). X^1 is a further adaptive asparagine to serine mutation.

Figures 4A and 4B illustrate a nucleotide sequence (SEQ ID NO: 4) encoding SEQ ID NO: 3. X^1 is A or G, G was found to create Ser adaptive mutation at amino acid 268 of NS5A.

DETAILED DESCRIPTION OF THE INVENTION

HCV replicons provide a cell culture system for measuring the ability of a compound to affect HCV replication. Compounds inhibiting HCV replication have research and therapeutic applications. Therapeutic applications include using those compounds having appropriate pharmacological properties such as efficacy and lack of unacceptable toxicity to treat or inhibit onset of HCV in a patient.

Replicons containing NS5B from genotype 2b can be used by themselves to measure the effect of a compound on HCV activity and can used as part of a panel of different replicons to evaluate the activity of a compound against HCV NS5B present in different HCV isolates. For example, a compound targeting NS5B can be tested using a first replicon containing NS3-NS5B of genotype 1b and second replicon produced from the first replicon where at least substantially all the genotype 1b NS5B sequence is replaced with a NS5B genotype 2b sequence.

The identification of genotype 2b NS5B adaptive mutations facilitates the use different genotype 2b NS5B sequences, including sequences obtained from clinical isolates. Replicons containing the NS5B sequences obtained from clinical isolates can be used to evaluate the effectiveness of a compound against different HCV isolates and provide an indication of the effectiveness of a compound in a particular individual.

I. Genotype 2b NS5B Sequence

Genotype 2b NS5B sequences described herein contain an amino acid sequence substantially similar to the amino acid sequence of SEQ ID NO: 1, wherein Z^1 is serine, X^1 is asparagine, X^2 is methionine, and X^3 is isoleucine ("prototype NS5B genotype 2b sequence"). Genotype 2b NS5B sequences include different naturally occurring sequences and modifications of naturally occurring sequences having a substantially similar sequence as the prototype NS5B genotype 2b sequence.

The prototype NS5B genotype 2b sequence provides a reference point for a genotype 2b NS5B. A sequence substantially similar to the prototype NS5B genotype 2b has a sequence identity of at least 90% to the prototype NS5B genotype 2b sequence. Percent identity is calculated by determining the number of amino acids within the test sequence that are identical to the reference sequence, dividing this number by the total number of residues, and then multiplying this fraction by 100. Amino acid alterations can be any combination of additions, deletions, or substitutions.

Figure 1 illustrates an amino acid sequence providing some examples of different amino acids of a NS5B genotype 2b (SEQ ID NO: 1). The underlined sequence represents a

sequence derived from a genotype 1b NS5B. Z^1 is threonine or serine. X^1 is asparagine or serine, where serine was identified as an adaptive mutation. X^2 is methionine or isoleucine, where isoleucine was identified as an adaptive mutation. X^3 is isoleucine or leucine, where leucine was identified as an adaptive mutation.

Preferred genotype 2b NS5B sequences contain one or more of the following adaptive mutations: 24S, 31I and 392L. More preferably, the genotype 2b NS5B sequence has at least a 31I adaptive mutation or a combination of adaptive mutations containing 24S and 392L. In an embodiment of the present invention the genotype 2b NS5B sequence is provided by SEQ ID NO: 1 containing one or more adaptive mutations described herein.

Adaptive mutations can be introduced into a sequence or it is possible that they may be present in a naturally occurring sequence. The HCV sequence has a high rate of sequence variability and mutation due to the error-prone mechanism of NS5B polymerase, which randomly mis-incorporates ribonucleotides at a rate of approximately 10^{-4}.

Figure 2 illustrates a nucleotide sequence (SEQ ID NO: 2) encoding the amino acid sequence of SEQ ID NO: 1. The underlined sequence represents a sequence from a genotype 1b NS5B sequence. X^1, X^2, X^3, Y^1,Y^2,Y^3, Y^4,Y^5, Y^6, Y^7, Y^8, and Y^9 are as described in the Brief Description of the Drawings *supra.*

Preferred genotype 2b NS5B encoding sequences provide one or more of the following adaptive mutations: 24S, 31S and 392L. More preferably, the genotype 2b NS5B sequence has at least a 31I adaptive mutation or a combination of adaptive mutations containing 24S and 392L.

In an embodiment of the present invention the genotype 2b NS5B encoding sequence is provided by SEQ ID NO: 2 containing one or more adaptive mutations described herein. For example, the genotype 2b NS5B encoding sequence is provided by SEQ ID NO: 2, wherein X^1 is A or G, X^2 is T, X^3 is A or C, Y^1 is A or T, Y^2 is C or A, Y^3 is A or T, Y^4 is A or C, Y^5 is A or G, Y^6 is C or G, Y^7 is T or C; Y^8 is G or C, and Y^9 is C or A; or X^1 is A, X^2 is T, X^3 is A, Y^1 is 'A or T, Y^2 is C or A, Y^3 is A or T, Y^4 is A or C, Y^5 is A or G, Y^6 is C or G, Y^7 is T or C; Y^8 is G or C, and Y^9 is C or A: or wherein X^1 is G, X^2 is G or T, X^3 is Y^1 is A or T, Y^2 is C or A, Y^3 is A or T, Y^4 is A or C, Y^5 is A or G, Y^6 is C or G, Y^7 is T or C; Y^8 is G or C, and Y^9 is C or A; or X^1 is A, X^2 is T, X^3 is A, Y^1 is A or T, Y^2 is C or A, Y^3 is A or T, Y^4 is A or C, Y^5 is A or G, Y^6 is C or G, Y^7 is T or C; Y^8 is G or C, and Y^9 is C or A.

Additional genotype 2b NS5B adaptive mutations can be selected for using techniques described herein. Examples of such techniques are provided in the Examples *infra.*

II. Chimeric Replicons

Chimeric replicons providing functional genotype 2b NS5B activity can be produced by combining a NS3-5A genotype 1b encoding sequence of a replicon with a genotype 2b NS5B encoding nucleic acid sequence. The genotype 2b NS5B encoding nucleic acid sequence can be enhanced as described in Section I *supra* and the Examples *infra*.

The basic subunit of an HCV replicon encodes a HCV NS3-5B polyprotein along with a suitable 5'-UTR-partial core (PC) region and 3'-UTR. Additional regions may be present including those coding for HCV proteins or elements such as the complete core, E1, E2, p7 or NS2; and those coding for other types of proteins or elements such as an encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), a reporter protein, or a selection protein.

The HCV 5'-UTR-PC region provides an IRES for protein translation and elements needed for replication. The HCV 5'-UTR-PC region includes naturally occurring HCV 5'-UTR extending about 36 nucleotides into a HCV core encoding region, and functional derivatives thereof. The IRES and PC can be present in different locations such as a site downstream from a sequence encoding a selection protein, a reporter protein, or an HCV polyprotein.

Functional derivatives of the 5'-UTR-PC region able to initiate translation and assist replication can be designed taking into account structural requirements for HCV translation initiation. (See, for example, Honda *et al.*, 1996. *Virology 222*, 31-42.) The effect of different modifications to a 5'-UTR-PC region can be determined using techniques measuring replicon activity.

In addition to the HCV 5'-UTR-PC region, other types of IRES elements can also be present in a replicon. Other types of IRES elements can be present in different locations including immediately upstream of the region encoding an HCV polyprotein. Examples of non-HCV IRES elements that can be used are the EMCV IRES, poliovirus IRES, and bovine viral diarrhea virus IRES.

The HCV 3'-UTR assists HCV replication. HCV 3'-UTR includes naturally occurring HCV 3'-UTR and functional derivatives thereof. Naturally occurring 3'-UTR's have a poly U tract and an additional region of about 100 nucleotides. (Tanaka *et al.*, 1996. *J. Virol. 70*, 3307-3312, Kolykhalov *et al.*, 1996. *J. Virol. 70*, 3363-3371.) At least *in vivo*, the 3'-UTR appears to be essential for replication. (Kolykhalov *et al.*, 2000. *J. Virol. 4*, 2046-2051.) Examples of naturally occurring 3' UTR derivatives are described by Bartenschlager, U.S. Patent No. 6,630,343.

The NS3-NS5B polyprotein encoding region provides for a polyprotein that can be processed in a cell into different proteins. Proper processing can be measured by assaying, for example, HCV protein production.

An HCV replicon may contain non-HCV sequences in addition to HCV sequences. The additional sequences should not prevent replication and expression, and preferably serve a useful function. Sequences that can be used to serve a useful function include a selection sequence, a reporter sequence, transcription elements, translation elements and a ribozyme to generate the authentic 3' end of the HCV RNA.

A selection sequence in a HCV replicon facilitates the identification and/or isolation of a cell containing the replicon. Selection sequences providing resistance to an agent that inhibits cell growth can be used in conjunction with selective pressure inhibiting growth of cells not containing the selection sequence. Examples of selection sequences include sequences encoding antibiotic resistance, and ribozymes; and reporters compatible with cell sorting such as green fluorescence protein and beta-lactamase.

Antibiotic resistance can be used in conjunction with an antibiotic to select for cells containing replicons. Examples of selection sequences providing antibiotic resistance are sequences encoding resistance to neomycin, hygromycin, puromycin, or zeocin.

A ribozyme serving as a selection sequence can be used in conjunction with an inhibitory nucleic acid molecule preventing cellular growth. The ribozyme recognizes and cleaves the inhibitory nucleic acid.

A reporter sequence can be used to detect replicon replication or protein expression. Preferred reporter proteins are enzymatic proteins whose presence can be detected by measuring product produced by the protein, or non-enzymatic proteins which can be measured directly. Examples of reporter proteins, both enzymatic and non-enzymatic, include luciferase, beta-lactamase, secretory alkaline phosphatase, beta-glucuronidase, and green fluorescent protein. In addition, a reporter nucleic acid sequence can be used to provide a reference sequence that can be targeted by a complementary nucleic acid probe. Hybridization of the complementary nucleic acid probe to its target can be determined using standard techniques.

Replicons containing reporter sequences may or may not also contain a selection sequence. Selection sequences providing resistance to an agent inhibiting cell growth can be used in conjunction with selective pressure to select for cells maintaining the replicon.

Additional sequences can be part of the same cistron as the HCV polyprotein or can be a separate cistron. If part of the same cistron, additional sequences coding for a protein

should result in a product that is either active as a chimeric protein or is cleaved inside a cell so it is separated from HCV protein.

Selection and reporter sequences encoding a protein when present as a separate cistron should be associated with elements needed for translation. Such elements include an IRES 5' to the selection or reporter sequence.

A preferred construct is a bicistronic replicon, where one cistron encodes a selection or reporter sequence and the second cistron encodes HCV proteins. More preferably, the first cistron contains a HCV 5'-UTR-PC region joined to the selection or reporter sequence; and the second cistron contains the EMCV internal ribosome entry site, NS2-NS5B or NS3-NS5B, and a 3'-UTR.

The production and use of replicons containing HCV genotype 1b NS3-5B sequences with adaptive mutations are well known in art. (See, for example, Lohmann *et al., Science 285*, 110-113, 1999, Blight *et al., Science 290,* 1972-1974, 2000, Lohmann *et al., Journal of Virology 75,* 1437-1449, 2001, Pietschmann *et al., Journal of Virology 75,* 1252-1264, 2001, Krieger *et al., J. of Virology 75*: 4614-4624, 2001, Bartenschlager, *Nat. Rev. Drug Discov. 1*(11): 911-916, 2002, Carroll *et al., J. Biological Chemistry, 278*: 11979-11984, 2003, Grobler et al., *J. of Biological Chemistry 278*:16741-16746, 2003, Murray *et al., J. of Virology 77*: 2928-2935, 2003, Vrolijk *et al., J. Virol. Methods 10*(2): 201-209, 2003, Lohmann *et al., J. Virol. 77*(5): 3007-3019, 2003, Bartenschlager, U.S. Patent No. 6,630,343, Rice *et al.,* International Publication Number WO 01/89364, published November 29, 2001, Bichko International Publication Number WO 02/238793, published May 16. 2002, Kukolj *et al.,* International Publication Number WO 02/052015, published July 4, 2002, De Francesco *et al.,* International Publication Number WO 02/059321, published August 1, 2002.)

SEQ ID NO: 3 provides an example of a genotype 1b NS3-NS5A amino acid from a replicon (Figure 3). X^1 is a further adaptive asparagine to serine mutation found to be useful in chimeric replicons.

SEQ ID NO: 4 provides a nucleotide sequence encoding SEQ ID NO: 3. (See Figures 4A and 4B).

III. Chimeric Replicon Production

Chimeric replicons can be produced by replacing substantially all of a NS5B sequence of a HCV replicon comprising a NS3-5B genotype 1b sequence with a genotype 2b NS5B encoding nucleic acid sequence. Replacing "substantially all" replaces a sufficient amount of the NS5B sequence such that the resulting sequence is a NS5B genotype 2b sequence. "Substantially all" indicates replacing the entire sequence or a portion of the entire sequence.

Replacement can be achieved by different techniques such as recombinant nucleic acid techniques used to modify a nucleic acid sequence and/or synthesis techniques used to produce a particular sequence. Techniques for altering nucleotides and synthesizing nucleotides are well known in the art. (Ausubel, *Current Protocols in Molecular Biology,* John Wiley, 1987-1998, and Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual,* 2nd Edition, Cold Spring Harbor Laboratory Press, 1989.)

Nucleic acid encoding a particular amino acid sequence can be obtained taking into account the genetic code. Amino acids are encoded by codons as follows:

A=Ala=Alanine: codons GCA, GCC, GCG, GCU
C=Cys=Cysteine: codons UGC, UGU
D=Asp=Aspartic acid: codons GAC, GAU
E=Glu=Glutamic acid: codons GAA, GAG
F=Phe=Phenylalanine: codons UUC, UUU
G=Gly=Glycine: codons GGA, GGC, GGG, GGU
H=His=Histidine: codons CAC, CAU
I=Ile=Isoleucine: codons AUA, AUC, AUU
K=Lys=Lysine: codons AAA, AAG
L=Leu=Leucine: codons UUA, UUG, CUA, CUC, CUG, CUU
M=Met=Methionine: codon AUG
N=Asn=Asparagine: codons AAC, AAU
P=Pro=Proline: codons CCA, CCC, CCG, CCU
Q=Gln=Glutamine: codons CAA, CAG
R=Arg=Arginine: codons AGA, AGG, CGA, CGC, CGG, CGU
S=Ser=Serine: codons AGC, AGU, UCA, UCC, UCG, UCU
T=Thr=Threonine: codons ACA, ACC, ACG, ACU
V=Val=Valine: codons GUA, GUC, GUG, GUU
W=Trp=Tryptophan: codon UGG
Y=Tyr=Tyrosine: codons UAC, UAU.

Replicon activity can be measured using techniques such those described in references dealing with adaptive mutations (Section II. *supra.*), and those described in the Examples *infra.*

In different embodiments, a chimeric replicon is produced encoding the genotype 1b NS3-NS5A provided by SEQ ID NO: 3, and the genotype 2b NS5B provided by SEQ ID NO: 1 containing one or more adaptive mutation enhancing NS5B activity; and the replicon comprises the genotype 1b NS3-NS5A nucleotide sequence provided by SEQ ID NO: 4, and the

genotype 2b NS5B provided by SEQ ID NO: 2 containing one or more adaptive mutation enhancing NS5B activity.

IV. Resistance Phenotyping

Resistance phenotyping can be performed to determine the effect of a particular compound on different HCV isolates. Resistance phenotyping can be defined as determining whether a mutation confers resistance to a compound of interest within the genetic background of a circulating variant obtained from a patient sample. The guidance provided herein can be employed to produce replicons containing different genotype 2b NS5B activities. For example, a genotype 2b NS5B can be isolated from a clinical sample, modified to contain a useful adaptive mutation, and then used to replace substantially all the NS5B of a genotype 1b replicon. The ability of a compound to effect chimeric replicon activity can be evaluated. Mutations that were demonstrated in cell culture to confer resistance to this compound can be engineered into this construct, and resistance determined in the genetic context of this variant.

V. Host Cells

Preferred cells for use with a HCV replicon are Huh-7 cells and Huh-7 derived cells. "Huh-7 derived cells" are cells produced starting with Huh-7 cells and introducing one or more phenotypic and/or genotypic modifications.

Huh-7 derived cells include replicon enhanced cells produced from Huh-7. Replicon enhanced cells can be obtained by introducing a replicon into a cell, selecting for cells supporting replicon activity, and completely or partially curing the cells of the replicon. The cured or partially cured cells can be used as a host for introducing another replicon. (De Francesco *et al.*, International Publication Number WO 02/059321, published August 1, 2002, Murray *et al., J. of Virology, 77*:2928-2935, 2003.)

VI. Detection Methods

Methods for detecting replicon activity include those measuring the production or activity of replicon RNA and encoded protein. Measuring can be by qualitative or quantitative analysis. Preferably, replicon activity is measured using a reporter protein.

Preferred reporters are beta-lactamases and luciferases. Beta-lactamases are enzymes catalyzing the cleavage of the beta-lactam ring present in cephalosporins. Different naturally occurring beta-lactamases and functional derivatives of naturally occurring beta-lactamases are well known in the art. (For example, see, Ambler, *Phil. Trans R. Soc. Lond.* Ser.

B. 1980. 289, 321-331, Kadonaga *et al.,* 1984. *J. Biol. Chem. 259,* 2149-2154, and U.S. Patent No. 5,744,320.)

Intracellular beta-lactamase activity is preferably measured using a fluorogenic substrate cleaved by beta-lactamase. Preferred substrates are membrane permeant fluorogenic substrates that become membrane impermeant inside a cell, and that are cleaved by beta-lactamase to produce a detectable signal. Examples of such substrates are provided in Zlokarnik *et al.,* 1998. *Science 279,* 84-88, and Tsien *et al.*, U.S. Patent No. 5,741,657.

Beta-lactamase activity can be measured, for example, using a cell-permeant fluorescent beta-lactamase substrate such as CCF2-AM or CCF4-AM (Aurora Biosciences, Inc., San Diego, CA). These substrates contain an ester group facilitating transport across the cell membrane. Inside the cell, the ester group is cleaved rendering the substrate membrane impermeant. The intact substrates when stimulated with light of ~405 nm, emit green fluorescence (~530 nm) due to resonant energy transfer from a coumarin to fluorescein dye molecule. Cleavage of the substrate by beta-lactamase disrupts the resonance energy transfer and, the fluorescence emission changes to a blue color (~460 nm) of only the coumarin. The fluorescence emissions of the substrate present in the cells can be detected by, for example, fluorescence microscopy or by a fluorometer in conjunction with appropriate emission and excitation filters.

Beta-lactamase inhibitors such as clavulanic acid can be used to enhance a beta-lactamase reporter system by being present throughout the assay. For example, clavulanic acid being present throughout an assay involving an HCV replicon beta-lactamase reporter sensitizes the assay towards HCV replication inhibitors.

Beta-lactamase activity can be measured, for example, by direct visualization of cells using a fluorescence microscope. Quantitation of HCV replication can be accomplished using a CCD camera acquiring digital images and suitable software quantitating the number of blue and green cells present in such images. This method quantitates the number of cells in a population harboring HCV replicons expressing beta-lactamase and this measurement is typically expressed as percentage blue cells (% Blue cells).

Another method for measuring beta-lactamase activity employs a fluorescence plate reader that quantitates the amount and green (~530 nm) or blue (~460 nm) fluorescence emitted by cells stimulated with light of ~405 nm. This method can be used for high throughput screening.

Quantitation of beta-lactamase activity can also be accomplished by FACS. This method quantitates the number of blue and green cells in a given cell population as well as the

amount of blue and green fluorescence. Instruments capable of cell sorting can be used to isolate cells harboring HCV replicons expressing beta-lactamase.

Techniques suitable for measuring RNA production include those detecting the presence or activity of RNA. RNA can be detected using, for example, complementary hybridization probes or quantitative PCR. Techniques for measuring hybridization between complementary nucleic acids and quantitative PCR are well known in the art. (See for example, Ausubel, *Current Protocols in Molecular Biology,* John Wiley, 1987-1998, Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual,* 2^{nd} Edition, Cold Spring Harbor Laboratory Press, 1989, and U.S. Patent No. 5,731,148.)

RNA enzymatic activity can be provided to the replicon by using a ribozyme sequence. Ribozyme activity can be measured using techniques detecting the ability of the ribozyme to cleave a target sequence.

Techniques measuring protein production include those detecting the presence or activity of a produced protein. The presence of a particular protein can be determined by, for example, immunological techniques. Protein activity can be measured based on the activity of an HCV protein or a reporter protein sequence.

Techniques for measuring HCV protein activity vary depending upon the protein that is measured. Techniques for measuring the activity of different non-structural proteins such as NS2/3, NS3, and NS5B, are well known in the art. (See, for example, references provided in the Background of the Invention.)

Assays measuring replicon activity also include those detecting virion production from a replicon producing a virion; and those detecting a cytopathic effect from a replicon producing proteins exerting such an effect. Cytopathic effects can be detected by assays suitable to measure cell viability.

Assays measuring replicon activity can be used to evaluate the ability of a compound to modulate HCV activities. Such assays can be carried out by providing one or more test compounds to a cell expressing a HCV replicon and measuring the effect of the compound on replicon activity. If a preparation containing more than one compound modulates replicon activity, individual compounds or smaller groups of compounds can be tested to identify replicon active compounds.

VII. Examples

Examples are provided below further illustrating different features of the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Example 1: Production of Genotype 2b NS5B sequence

A genotype 2b NS5b sequence was obtained by rescuing NS3-5B genotype 2b sequences from sera and producing a consensus sequence. The consensus sequence was based on the different sequences that were obtained.

Genotype 2b Sequences Rescue

The NS3-5B portion of the genomic RNA of HCV genotype 2b was rescued from infected chimp sera by RT/PCR. Briefly, viral RNA was isolated from 140 µl of sera using Qiagen's Viral RNA kit. Ten microliters of isolated RNA (1/5 of the total yield) was reverse transcribed into cDNA using Stratagene's ProStar kit and dA(34) as primer. After cDNA synthesis, the RNA was degraded using 1 µl of RNAses H (1 U/µl) and T1 (100 U/µl) (both from Roche), at 37°C for 20 minutes. The reaction was heat inactivated at 65°C for 20 minutes prior to PCR.

PCR was performed using 5 µl of the RT reaction and Roche's Expand Long PCR kit. For PCRI, primers 5' ATGGAGAAGAAGGTCATTGTGTG and dA(34) were used as the forward and reverse primers, respectively. A second, nested PCR was then performed with 10 µl of PCRI, forward primer 5' GCTCCCATTACTGCCTACACTCA, and reverse primer 5' CCGCTCTACCGAGCGGGGAGT. PCR reactions were conducted in a Biometra T-gradient cycler, cycling conditions 94°C for 2 minutes, then 15 cycles at 94°C for 15 seconds, 56°C for 40 seconds, then 68°C for 6.5 minutes, followed by 20 cycles of the same, with a 20 second auto extension added onto each cycle.

The rescued NS3-5B DNA fragment was recovered from a 1% agarose TAE gel using Qiagen's gel extraction kit, and was cloned into Novagen's pSTBlue-1 perfectly blunt cloning system. Individual bacterial colonies were propagated for DNA and sequenced using an ABI 3100 DNA sequencer. Sequences were analyzed with Sequencher (Gene Codes Corp.).

NS5B Consensus Sequence

A consensus NS5B sequence was derived from the sequence of five independent NS3-5B clones. The clone with the fewest NS5B changes from the consensus was used as a template to generate the consensus clone through site-directed mutagenesis. The template NS5B encoding sequence was altered to encode for M229T and A558G substitutions.

Site-directed mutagenesis was performed on a vector generated by subcloning a genotype 2b NS5B gene into pJG1062. Subcloning was achieved by generating a PCR product using the NS5B encoding sequence, digesting with Bcl I and Cla I, and subcloning into the BclI-

ClaI sites of vector pJG1062. pJG1062 encodes a BamHI-XbaI fragment of con1. (Grobler *et al., J. of Biological Chemistry 278*:16741-16746, 2003.)

Example 2: Chimeric HCV Replicon Production

Chimeric HCV replicon were produced containing a NS3-5A genotype 1b based a modified BK (Grobler *et al., J. of Biological Chemistry. 278*:16741-16746, 2003) and a NS5B genotype 2b based sequence. Replicon production involved producing a chimeric construct by joining a NS3-5A genotype 1b sequence, a NS5B genotype 2b sequence, and a neomycin resistant sequence; infecting Huh7 cells with the chimeric construct; and selecting for neomycin resistant colonies. Characterization of neomycin resistant colonies identified three different adaptive mutations.

Chimeric Construct Production

A chimeric construct was produced by replacing substantially all of a NS5B sequence of a NS3-5B genotype 1b replicon with the NS5B genotype 2b sequence. The mutated genotype 2b NS5B encoding sequence from Example 1 was subcloned into pJG1185 as a BclI-ClaI fragment. pJG1185 encodes a subgenomic genotype 1b replicon based on HCV BK with adaptive mutations. (Grobler *et al., J. of Biological Chemistry. 278*:16741-16746, 2003.)

The resulting chimeric construct replaced the genotype 1b NS5B with the NS5B genotype 2b clone starting at amino acid residue 11 of NS5B. The clone was designated clone "bla-2b". Clone "neo-2b" was produced by replacing the bla-2b β-lactamase gene with a neomycin phosphotransferase (Neo^r) encoding gene. Clone con1-2bM31I discussed in example 4 is similar to bla-2bM31I except that the NS3-5A replicon sequences are Con1b sequences. This was constructed by cloning the genotype 2b NS5B with M31I as a BclI-ClaI fragment into vector pJG1073. pJG1073 encodes a subgenomic genotype 1b replicon with NS3 through NS5A based on HCV con1. (Grobler *et al., J. of Biological Chemistry. 278*:16741-16746, 2003.)

Selection and Characterization of Neo Resistant Colonies

Neo-2b was used as a starting point to select for adaptive mutations. To generate RNA for transfection, plasmids were linearized by digestion of an XbaI site distal to the replicon sequences. RNA was generated using MEGAscript™ (Ambion, Austin, TX) and quantitated by UV absorbance.

Huh-7 cells were seeded at a density of 300,000 cells per well in a 6 well cluster plate 16 hours prior to transfection. Transfection was accomplished using Optimem I and DMRIE-C reagents (Invitrogen Life Technologies) as described by Murray *et al., J. of Virology,*

77:2928-2935, 2003, except 2.5 μg of RNA was used per well for transfection of cells. The day after transfection, cells were split into T150 flasks with peel off tops (TKR Biotech) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, nonessential amino acids, 2 mM GlutaMAX, and Penn/Strep (Invitrogen Corp., Carlsbad, CA) containing 250 μg/ml Geneticin (Invitrogen) and a 1/500 dilution (v/v) of Fungizone (Invitrogen). Cells were kept sub-confluent over the course of the selection by expanding into fresh media until neo resistant colonies arose.

Three colonies were obtained and further characterized. The colonies were trypsinized in cloning cylinders (Sigma), then expanded ultimately to T225 flasks. Twenty percent of a T225 harvest (~ 1-2 x 10^6 cells) was used in RT/PCR analysis. RNA was isolated from the resistant cell lines using Qiagen's RNA Easy kit. Ten micrograms of isolated RNA was taken for RT using the dA(34) primer as described above. The RT reaction was treated with RNAses as described earlier, and 5 μl was used for PCR using Novagen's KOD hot start polymerase. Five overlapping, nested, PCR rescues (designated A-E) were performed to recover the NS3-5B region. PCR primers for the fragments were as follows:

A: 5' CTCTCCTCAAGCGTATTCAACAAGG forward
5' CCGTGCAGCGTAGGTTTCAGCCGTA reverse

5' CCCATTGTATGGGATCTGATCTGG forward
5' CAAGCTGAAGTCGACTGTCTGGGTGACA reverse

B: 5' TACTTGGTCACGAGACATGCTGACGTCAT forward
5' GGAGAGGATAGCAGGGAGT reverse

5' CGTATATGTCTAAGGCACACGGTATTGAC forward
5' GGCTGGTGATAGAGGCTGTGAATGCCAT reverse

C: 5' GGATCAAATGTGGAAGTGTCTCATACGG forward
5' TCGAGGTTGTGGAGTACAC reverse

5' GCAATAGCATCATTGATGGCATTCACAGC forward
5' GGCCTCGATGAGGTCAGCGT reverse

D: 5' CTCTCCTCAAGCGTATTCAACAAGG
5' d(A34)

5' GTAAAGTGCCCGTGTCAGGT
5' CATGATAGTTGTGTCAATTGG reverse

E: 5' GTCTACCGTGAGCGAGGAA
5' d(A34) reverse

5' ATACTCCTGGACAGGGGCCCT
5' GCGCGCGCATCGATCGGGGAGTAAAAAGATGCCTAC

For every set of reactions, the PCR I primers are the first pair in each set, while the PCRII primers are the second. For PCRII, 5 μl of the PCRI reaction was used. Generally, the cycling conditions for PCR were 94° C 2 minutes, then 94°C for 15 seconds, 55°C for 40 seconds, and 68°C for 2.5 minutes, for 35 cycles.

The PCRII fragments were size fractionated on 1% agarose TAE gels and isolated using Qiagen's gel extraction kit. The fragments were sequenced directly on an ABI 3100 sequencer and analyzed with Sequencher.

Each of the three different surviving cells were found to contain an adaptive mutation conferring replication competence in a chimera construct. The adaptive mutations were N24S, M31I, and I392L in the prototype NS5B 2b sequence.

Example 3: Establishment of Persistently Replicating Cell Lines and Addition of Test Compounds

Selection of persistently replicating cells lines expressing β-lactamase, using bla:2b chimeric replicons each harboring a different mutation (N24S, M31I, I392L) defined in the Neo^r selection studies described above, was performed in the enhanced replication Huh7 derived cell line MR2. (Murray *et al., J. of Virology, 77*:2928-2935, 2003.) To generate RNA for transfection, plasmids were linearized by digestion of an XbaI site distal to the replicon sequences. RNA was generated using MEGAscript™ (Ambion, Austin, TX) and quantitated by UV absorbance.

MR2 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, nonessential amino acids, 2 mM GlutaMAX, and Penn/Strep (Invitrogen Corp., Carlsbad, CA). Cells were seeded the night prior to transfection at

a density of 300,000 cells per well in a 6-well dish. A mixture of 5 μg RNA, 12 μl of DMRE-C (Invitrogen Corp., Carlsbad, CA), and 2 ml of Opti-Mem (Invitrogen Corp., Carlsbad, CA) media was prepared and allowed to sit for 10 minutes. During this time media was removed from the MR2 cells, cells were washed once with Opti-Mem, then the RNA:DMRE-C mixture was added dropwise, and incubated for ~8 hours. Transfection media was removed by aspiration, and replaced with 2 ml DMEM. Transfected cells continued to grow overnight.

The next morning cells were expanded into two T75 flasks and grown for an additional three days. A duplicate well for each replicon construct was analyzed for β-lactamase activity to verify that the transfection efficiency was > 90%.

On day 4 cells from one flask were collected, counted, and seeded into a 96 well Cytostar plate at a density of 7,500 cells/well in the presence of 1 μM clavulonic acid. The appropriate dilution of an NS5B inhibitor was added immediately in an equal volume, thus adjusting the final clavulonic acid concentration to 0.5 μM. Simultaneously, the other flasks were analyzed for β-lactamase activity to verify both that activity from a non-replicating control sub-genomic replicon was undetectable, and establish the day 4 replication activity of the test replicons. The non-replicating control, con1:GAA, is non-functional due to an Asp-to-Ala substitution of NS5B (wild-type is GDD). Cells with drug were incubated for two days, and analyzed as described in Example 4.

Example 4: Analysis of β-lactamase Activity

Medium was removed by aspiration and cells were stained for 2 hours with CCF4-AM (Invitrogen Corp., Carlsbad, CA) in DMEM supplemented with 25 mM HEPES, pH 8.0. Fluorescence due to β-lactamase activity was measured through excitation at 405 nm, followed by measurement of the emission at 460 nm using a CytoFluor 4000 fluorescence plate reader. Equal cell count and viability was verified by measuring the emission at 530 nm. A tolerance limit of 30% per data set was accepted.

EC_{50} determinations for 2'C-methyladenosine were calculated as a percent of the DMSO control by fitting the data to a 4 parameter fit function using Kaleidagraph software (Synergy Software, Reading, PA). The results are shown in Table 1.

Table 1

	Con1-2bM31I	BK-2bM31I
EC_{50}	407 nM	667 nM
Slope	1.3	1.9

"Con1" indicates NS3-5A and the first 11 NS5B amino acids, of a modified Con1b (Lohmann *et al.*, *Science 285*, 110-113, 1999). "BK" indicates NS3-NS5A and the first 11 NS5B amino acids, of a modified BK (Grobler *et al.*, *J. of Biological Chemistry. 278*:16741-16746, 2003). "2bM31I" indicates an adaptive mutation made to the prototype NS5B genotype 2b sequence.

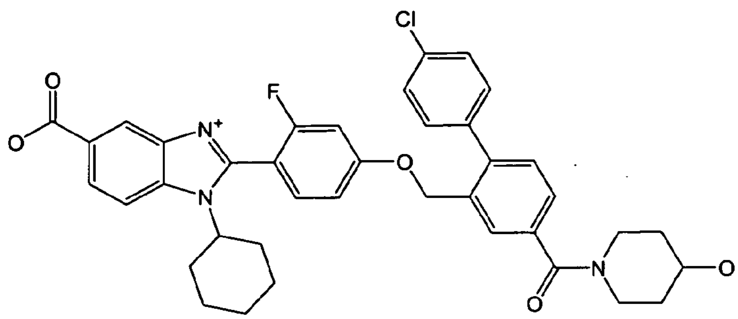
Two bk:2B bla chimeric replicons bearing changes at residues 24 and 392 were not robust enough by themselves for EC_{50} determination, although limited replication which could be inhibited at the EC_{95} for 2'C-methyladenosine could be demonstrated. The combination of these mutations enhanced replication to a level sufficient for drug titration when present in the same construct. In addition, 24S within NS5B, when coupled with a serine at residue 268 of NS5A (within the BK replicon) also supported drug-titratable replication. (See Table 2.)

Table 2

	EC_{50}	Slope
Con1-2bM31I	407 nM	1.3
BK-2bM31I	667 nM	1.9
BK NS5a268S-2bN24S	982 nM	0.81
BK-2b N24S/L392I	185 nM	0.56

"Con1" "BK" and "2bM31I" are as described in Table 1. "NS5a268S" indicates an additional adaptive mutation to "BK" resulting in a serine at position 268 of NS5A. "2bN24S" indicates an adaptive mutation made to the prototype NS5B genotype 2b sequence. "N24S/L392I" indicates adaptive mutations made to the prototype NS5B genotype 2b sequence

There was no titratable inhibition by compound A in an 8 point titration (3 fold dilutions) from 20 μM to 9 nM (~ 10% inhibition for both the "Con1" and "BK" versions at 20 μM) for any of the BK:2B or con1:2B constructs described above. Compound A inhibited replication of genotype 1B replicons at sub-micromolar levels. Compound A has the following structure:



Other embodiments are within the following claims. While several embodiments have been shown and described, various modifications may be made without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED IS:

1. A method of enhancing the ability of a genotype 2b NS5B sequence to function in a replicon comprising the step of altering said genotype 2b NS5B sequence to encode one or more adaptive mutations selected from the group consisting of:
serine corresponding to position 24 of SEQ ID NO: 1;
isoleucine corresponding to position 31 of SEQ ID NO: 1; and
leucine corresponding to position 392 of SEQ ID NO: 1.

2. The method of claim 1, wherein altering said genotype 2b NS5B sequence comprises at least isoleucine corresponding to position 31 of SEQ ID NO: 1, or at least serine corresponding to position 24 of SEQ ID NO: 1 in combination with leucine corresponding to position 392 of SEQ ID NO: 1.

3. The method of claim 2, wherein said genotype 2b NS5B sequence is obtained from a clinical isolate.

4. A method of producing a chimeric replicon comprising the step of replacing substantially all of a NS5B sequence of a HCV replicon comprising a NS3-5B genotype 1b sequence with a genotype 2b NS5B encoding nucleic acid sequence.

5. The method of claim 4, further comprising the step of enhancing the ability of said genotype 2b NS5B according to the method of claim 1, wherein said enhancing can be done prior to, or after, said replacing step.

6. The method of claim 5, wherein said genotype 2b NS5B sequence is obtained from a clinical isolate.

7. A chimeric replicon comprising:
a) an NS3-5A sequence of a genotype 1b replicon; and
b) a genotype 2b NS5B encoding nucleic acid sequence.

8. The chimeric replicon of claim 7, further comprising a reporter or selection sequence.

9. The chimeric replicon of claim 8, wherein said genotype 2b NS5B sequence encodes at least one of the following amino acids in the indicated position:
serine corresponding to position 24 of SEQ ID NO: 1;
isoleucine corresponding to position 31 of SEQ ID NO: 1; and
leucine corresponding to position 392 of SEQ ID NO: 1.

10. The chimeric replicon of claim 9, wherein said genotype 2b NS5B sequence encodes least isoleucine corresponding to position 31 of SEQ ID NO: 1, or serine corresponding to position 31 of SEQ ID NO: 1 in combination with leucine corresponding to position 31 of SEQ ID NO: 1.

11. The chimeric replicon of claim 10, wherein said genotype 2b NS5B sequence encodes NS5B provided by SEQ ID NO: 1.

12. The chimeric replicon of claim 1, wherein said NS3-5A sequence consists of SEQ ID NO: 4 and NS5B sequence consists of SEQ ID NO: 2.

13. The chimeric replicon of claim 12, wherein for SEQ ID NO: 4 the nucleotide at position 268 is G.

14. A recombinant cell comprising a replicon made the method of any one of claims 2-6, or the replicon of any one of claim 7-13, wherein said replicon is expressed in said cell.

15. A method of measuring the ability of a compound to inhibit replicon activity comprising the steps of:

a) providing said compound to the recombinant cell of claim 12, and

b) measuring the ability of said compound to affect replicon activity.

ABSTRACT OF THE DISCLOSURE

The present invention features methods for enhancing the ability of a genotype 2b NS5B sequence to function in a replicon, for producing replicons containing a functional genotype 2b NS5B, and for using replicons to measure the ability of a compound to affect HCV replication that is sustained with the genotype 2b RNA polymerase. The ability to produce replicons containing a functional genotype 2b NS5B is illustrated by the production of chimeric replicons based on HCV genotype 1b where substantially all the NS5B sequence is replaced with a genotype 2b NS5B.

SMSY$\mathbf{Z}^1$WTGALITPCGPEEEKLPI$\mathbf{X}^1$PLSNSL$\mathbf{X}^2$RFHNKVYSTTSRSASLRAKKVTFDRVQVL
DAHYDSVLQDVKRAASKVSARLLTVEEACALTPPHSAKSRYGFGAKEVRSLSRRAVNHIRSV
WEDLLEDQHTPIDTTIMAKNEVFCIDPTKGGKKPARLIVYPDLGVRVCEKMALYDIAQKLPK
AIMGPSYGFQYSPAERVDFLLKAWGSKKDPMGFSYDTRCFDSTVTERDIRTEESIYQACSLP
QEARTVIHSLTERLYVGGPMTNSKGQSCGYRRCRASGVFTTSMGNTMTCYIKALAACKAAGI
VDPVMLVCGDDLVVISESQGNEEDERNLRAFTEAMTRYSAPPGDLPRPEYDLELITSCSSNV
SVALDSRGRRRYFLTRDPTTP$\mathbf{X}^3$TRAAWETVRHSPVNSWLGNIIQYAPTIWVRMVIMTHFFSI
LLAQDTLNQNLNFEMYGAVYSVNPLDLPAIIERLHGLEAFSLHTYSPHELSRVAATLRKLGA
PPLRAWKSRARAVRASLIAQGARAAICGRYLFNWAVKTKLKLTPLPEASRLDLSGWFTVGAG
GGDIYHSVSHARPRLLLLCLLLLSVGVGIFLLPDR

Fig. 1

TC$\mathbf{Y}^1$ATGTC$\mathbf{Y}^2$TAC$\mathbf{Y}^3$C$\mathbf{Y}^4$TGGAC$\mathbf{Y}^5$GG$\mathbf{Y}^6$GCC$\mathbf{Y}^7$T$\mathbf{Y}^8$AT$\mathbf{Y}^9$ACACCATGTGGGCCCGAAGAGGA
GAAGTTACCGATCA$\mathbf{X}^1$CCCTCTGAGTAATTCGCTCAT$\mathbf{X}^2$CGGTTCCATAATAAGGTGTACTCC
ACAACCTCGAGGAGTGCCTCTCTGAGGGCAAAGAAGGTGACTTTTGACAGGGTGCAGGTGCT
GGACGCACACTATGACTCAGTCTTGCAGGACGTTAAGCGGGCCGCCTCTAAGGTTAGTGCGA
GGCTCCTCACGGTAGAGGAAGCCTGCGCGCTGACCCCGCCCCACTCCGCCAAATCGCGATAC
GGATTTGGGGCAAAAGAGGTGCGCAGCTTATCTAGGAGGGCCGTTAACCACATCCGGTCCGT
GTGGGAGGACCTCCTGGAAGACCAACATACCCCAATTGACACAACTATCATGGCTAAAAATG
AGGTGTTCTGCATTGATCCAACTAAAGGTGGGAAAAAGCCAGCTCGCCTCATCGTATACCCC
GACCTTGGGGTCAGGGTGTGCGAAAAGATGGCCCTCTATGACATCGCACAAAAGCTTCCCAA
AGCGATAATGGGGCCATCCTATGGGTTCCAATACTCTCCCGCAGAACGGGTCGATTTCCTCC
TCAAAGCTTGGGGAAGTAAGAAGGACCCAATGGGGTTCTCGTATGACACCCGCTGCTTTGAC
TCAACCGTCACGGAGAGGGACATAAGAACAGAAGAATCCATATATCAGGCTTGTTCTCTGCC
TCAAGAAGCCAGAACTGTCATACACTCGCTCACTGAGAGACTTTACGTAGGAGGGCCCATGA
CAAACAGCAAAGGGCAATCCTGCGGCTACAGGCGTTGCCGCGCAAGCGGTGTTTTCACCACC
AGCATGGGGAATACCATGACATGTTACATCAAAGCCCTTGCAGCGTGTAAGGCTGCAGGGAT
CGTGGACCCTGTTATGTTGGTGTGTGGAGACGACCTGGTCGTCATCTCAGAGAGCCAAGGTA
ACGAGGAGGACGAGCGAAACCTGAGAGCTTTCACGGAGGCTATGACCAGGTATTCCGCCCCT
CCCGGTGACCTTCCCAGACCGGAATATGACTTGGAGCTTATAACATCCTGCTCCTCAAACGT
ATCGGTAGCGCTGGACTCTCGGGGTCGCCGCCGGTACTTCCTAACCAGAGACCCTACCACTC
CA$\mathbf{X}^3$TCACCCGAGCTGCTTGGGAAACAGTAAGACACTCCCCTGTCAATTCTTGGCTGGGCAAC
ATCATCCAGTACGCCCCCACAATCTGGGTCCGGATGGTCATAATGACTCACTTCTTCTCCAT
ACTATTGGCCCAGGACACTCTGAACCAAAATCTCAATTTTGAGATGTACGGGGCAGTATACT
CGGTCAATCCATTAGACCTACCGGCCATAATTGAAAGGCTACATGGGCTTGAAGCCTTTTCA
CTGCACACATACTCTCCCCACGAACTCTCACGGGTGGCAGCAACTCTCAGAAAACTTGGAGC
GCCTCCCCTTAGAGCGTGGAAGAGTCGGGCGCGTGCCGTGAGAGCTTCACTCATCGCCCAAG
GAGCGAGGGCGGCCATTTGTGGCCGCTACCTCTTCAACTGGGCGGTGAAAACAAAGCTCAAA
CTCACTCCATTGCCCGAGGCGAGCCGCCTGGATTTATCCGGGTGGTTCACCGTGGCGCCGG
CGGGGGCGACATTTATCACAGCGTGTCGCATGCCCGACCCCGCCTATTACTCCTTTGCCTAC
TCCTACTTAGCGTAGGAGTAGGCATCTTTTTACTCCCCGATCGATGA

Fig. 2

MAPITAYSQQTRGLLGCIITSLTGRDKNQVEGEVQVVSTATQSFLATCVNGVCWTVYHGAGS
KTLAGPKGPITQMYTNVDQDLVGWQAPPGARSLTPCTCGSSDLYLVTRHADVIPVRRRGDSR
GSLLSPRPVSYLKGSSGGPLLCPSGHAVGIFRAAVCTRGVAKAVDFVPVESMETTMRSPVFT
DNSSPPAVPQTFQVAHLHAPTGSGKSTKVPAAYAAQGYKVLVLNPSVAATLGFGAYMSKAHG
IDPNIRTGVRTITTGAPVTYSTYGKFLADGGCSGGAYDIIICDECHSTDSTTILGIGTVLDQ
AETAGARLVVLATATPPGSVTVPHPNIEEVALSNTGEIPFYGKAIPIEAIRGGRHLIFCHSK
KKCDELAAKLSGLGINAVAYYRGLDVSVIPTIGDVVVVATDALMTGYTGDFDSVIDCNTCVT
QTVDFSLDPTFTIETTTVPQDAVSRSQRRGRTGRGRMGIYRFVTPGERPSGMFDSSVLCECY
DAGCAWYELTPAETSVRLRAYLNTPGLPVCQDHLEFWESVFTGLTHIDAHFLSQTKQAGDNF
PYLVAYQATVCARAQAPPPSWDQMWKCLIRLKPTLHGPTPLLYRLGAVQNEVTLTHPITKYI
MACMSADLEVVTSTWVLVGGVLAALAAYCLTTGSVVIVGRIILSGRPAIVPDREFLYQEFDE
MEECASHLPYIEQGMQLAEQFKQKALGLLQTATKQAEAAAPVVESKWRALETFWAKHMWNFI
SGIQYLAGLSTLPGNPAIASLMAFTASITSPLTTQSTLLFNILGGWVAAQLAPPSAASAFVG
AGIAGAAVGSIGLGKVLVDILAGYGAGVAGALVAFKVMSGEMPSTEDLVNLLPAILSPGALV
VGVVCAAILRRHVGPGEGAVQWMNRLIAFASRGNHVSPTHYVPESDAAARVTQILSSLTITQ
LLKRLHQWINEDCSTPCSGSWLRDVWDWICTVLTDFKTWLQSKLLPQLPGVPFFSCQRGYKG
VWRGDGIMQTTCPCGAQITGHVKNGSMRIVGPKTCSNTWHGTFPINAYTTGPCTPSPAPNYS
RALWRVAAEEYVEVTRVGDFHYVTGMTTDNVKCPCQVPAPEFFTEVDGVRLHRYAPACRPLL
REEVTFQVGLNQYLVGSQLPCEPEPDVAVLTSMLTDPSHITAETAKRRLARGSPPSLASSSA
IQLSAPSLKATCTTHHVSPDADLIEANLLWRQEMGG$\mathbf{X}^1$ITRVESENKVVVLDSFDPLRAEEDE
REVSVPAEILRKSKKFPAAMPIWARPDYNPPLLESWKDPDYVPPVVHGCPLPPIKAPPIPPP
RRKRTVVLTESSVSSALAELATKTFGSSESSAVDSGTATALPDQASDDGDKGSDVESYSSMP
PLEGEPGDPDLSDGSWSTVSEEASEDVVCC

Fig. 3

ATGGCGCCCATCACGGCCTACTCCCAACAGACGCGGGGCCTACTTGGTTGCATCATCACTAG
CCTTACAGGCCGGGACAAGAACCAGGTCGAGGGAGAGGTTCAGGTGGTTTCCACCGCAACAC
AATCCTTCCTGGCGACCTGCGTCAACGGCGTGTGTTGGACCGTTTACCATGGTGCTGGCTCA
AAGACCTTAGCCGGCCCAAAGGGGCCAATCACCCAGATGTACACTAATGTGGACCAGGACCT
CGTCGGCTGGCAGGCGCCCCCCGGGGCGCGTTCCTTGACACCATGCACCTGTGGCAGCTCAG
ACCTTTACTTGGTCACGAGACATGCTGACGTCATTCCGGTGCGCCGGCGGGGCGACAGTAGG
GGGAGCCTGCTCTCCCCCAGGCCTGTCTCCTACTTGAAGGGCTCTTCGGGTGGTCCACTGCT
CTGCCCTTCGGGGCACGCTGTGGGCATCTTCCGGGCTGCCGTATGCACCCGGGGGGTTGCGA
AGGCGGTGGACTTTGTGCCCGTAGAGTCCATGGAAACTACTATGCGGTCTCCGGTCTTCACG
GACAACTCATCCCCCCCGGCCGTACCGCAGACATTTCAAGTGGCCCACCTACACGCTCCCAC
TGGCAGCGGCAAGAGTACTAAAGTGCCGGCTGCATATGCAGCCCAAGGGTACAAGGTGCTCG
TCCTCAATCCGTCCGTTGCCGCTACCTTAGGGTTTGGGGCGTATATGTCTAAGGCACACGGT
ATTGACCCCAACATCAGAACTGGGGTAAGGACCATTACCACAGGCGCCCCCGTCACATACTC
TACCTATGGCAAGTTTCTTGCCGATGGTGGTTGCTCTGGGGGCGCTTATGACATCATAATAT
GTGATGAGTGCCATTCAACTGACTCGACTACAATCTTGGGCATCGGCACAGTCCTGGACCAA
GCGGAGACGGCTGGAGCGCGGCTTGTCGTGCTCGCCACCGCTACGCCTCCGGGATCGGTCAC
CGTGCCACACCCAAACATCGAGGAGGTGGCCCTGTCTAATACTGGAGAGATCCCCTTCTATG
GCAAAGCCATCCCCATTGAAGCCATCAGGGGGGGAAGGCATCTCATTTTCTGTCATTCCAAG
AAGAAGTGCGACGAGCTCGCCGCAAAGCTGTCAGGCCTCGGAATCAACGCTGTGGCGTATTA
CCGGGGGCTCGATGTGTCCGTCATACCAACTATCGGAGACGTCGTTGTCGTGGCAACAGACG
CTCTGATGACGGGCTATACGGGCGACTTTGACTCAGTGATCGACTGTAACACATGTGTCACC
CAGACAGTCGACTTCAGCTTGGATCCCACCTTCACCATTGAGACGACGACCGTGCCTCAAGA
CGCAGTGTCGCGCTCGCAGCGGCGGGGTAGGACTGGCAGAGGTAGGATGGGCATCTACAGGT
TTGTGACTCCGGGAGAACGGCCCTCGGGCATGTTCGATTCCTCGGTCCTGTGTGAGTGCTAT
GACGCGGGCTGTGCTTGGTACGAGCTCACCCCCGCCGAGACCTCGGTTAGGTTGCGGGCCTA
CCTGAACACACCAGGGTTGCCCGTTTGCCAGGACCACCTGGAGTTCTGGGAGAGTGTCTTCA
CAGGCCTCACCCACATAGATGCACACTTCTTGTCCCAGACCAAGCAGGCAGGAGACAACTTC
CCCTACCTGGTAGCATACCAAGCCACGGTGTGCGCCAGGGCTCAGGCCCCACCTCCATCATG
GGATCAAATGTGGAAGTGTCTCATACGGCTGAAACCTACGCTGCACGGGCCAACACCCTTGC
TGTACAGGCTGGGAGCCGTCCAAAATGAGGTCACCCTCACCCACCCCATAACCAAATACATC
ATGGCATGCATGTCGGCTGACCTGGAGGTCGTCACTAGCACCTGGGTGCTGGTGGGCGGAGT
CCTTGCAGCTCTGGCCGCGTATTGCCTGACAACAGGCAGTGTGGTCATTGTGGGTAGGATTA
TCTTGTCCGGGAGGCCGGCTATTGTTCCCGACAGGGAGTTTCTCTACCAGGAGTTCGATGAA
ATGGAAGAGTGCGCCTCGCACCTCCCTTACATCGAGCAGGGAATGCAGCTCGCCGAGCAATT
CAAGCAGAAAGCGCTCGGGTTACTGCAAACAGCCACCAAACAAGCGGAGGCTGCTGCTCCCG
TGGTGGAGTCCAAGTGGCGAGCCCTTGAGACATTCTGGGCGAAGCACATGTGGAATTTCATC
AGCGGGATACAGTACTTAGCAGGCTTATCCACTCTGCCTGGGAACCCCGCAATAGCATCATT
GATGGCATTCACAGCCTCTATCACCAGCCCGCTCACCACCCAAAGTACCCTCCTGTTTAACA
TCTTGGGGGGGTGGGTGGCTGCCCAACTCGCCCCCCCCAGCGCCGCTTCGGCTTTCGTGGGC
GCCGGCATCGCCGGTGCGGCTGTTGGCAGCATAGGCCTTGGGAAGGTGCTTGTGGACATTCT
GGCGGGTTATGGAGCAGGAGTGGCCGGCGCGCTCGTGGCCTTCAAGGTCATGAGCGGCGAGA
TGCCCTCCACCGAGGACCTGGTCAATCTACTTCCTGCCATCCTCTCTCCTGGCGCCCTGGTC
GTCGGGGTCGTGTGTGCAGCAATACTGCGTCGACACGTGGGTCCGGGAGAGGGGGCTGTGCA
GTGGATGAACCGGCTGATAGCGTTCGCCTCGCGGGGTAATCATGTTTCCCCCACGCACTATG
TGCCTGAGAGCGACGCCGCAGCGCGTGTTACTCAGATCCTCTCCAGCCTTACCATCACTCAG
CTGCTGAAAAGGCTCCACCAGTGGATTAATGAAGACTGCTCCACACCGTGTTCCGGCTCGTG
GCTAAGGGATGTTTGGGACTGGATATGCACGGTGTTGACTGACTTCAAGACCTGGCTCCAGT
CCAAGCTCCTGCCGCAGCTACCGGGAGTCCCTTTTTTCTCGTGCCAACGCGGGTACAAGGGA
GTCTGGCGGGGAGACGGCATCATGCAAACCACCTGCCCATGTGGAGCACAGATCACCGGACA
TGTCAAAAACGGTTCCATGAGGATCGTCGGGCCTAAGACCTGCAGCAACACGTGGCATGGAA

Fig. 4A

CATTCCCCATCAACGCATACACCACGGGCCCCTGCACACCCTCTCCAGCGCCAAACTATTCT
AGGGCGCTGTGGCGGGTGGCCGCTGAGGAGTACGTGGAGGTCACGCGGGTGGGGGATTTCCA
CTACGTGACGGGCATGACCACTGACAACGTAAAGTGCCCATGCCAGGTTCCGGCTCCTGAAT
TCTTCACGGAGGTGGACGGAGTGCGGTTGCACAGGTACGCTCCGGCGTGCAGGCCTCTCCTA
CGGGAGGAGGTTACATTCCAGGTCGGGCTCAACCAATACCTGGTTGGGTCACAGCTACCATG
CGAGCCCGAACCGGATGTAGCAGTGCTCACTTCCATGCTCACCGACCCCTCCCACATCACAG
CAGAAACGGCTAAGCGTAGGTTGGCCAGGGGGTCTCCCCCCTCCTTGGCCAGCTCTTCAGCT
ATCCAGTTGTCTGCGCCTTCCTTGAAGGCGACATGCACTACCCACCATGTCTCTCCGGACGC
TGACCTCATCGAGGCCAACCTCCTGTGGCGGCAGGAGATGGGCGGGA**X**^{1}CATCACCCGCGTGG
AGTCGGAGAACAAGGTGGTAGTCCTGGACTCTTTCGACCCGCTTCGAGCGGAGGAGGATGAG
AGGGAAGTATCCGTTCCGGCGGAGATCCTGCGGAAATCCAAGAAGTTCCCCGCAGCGATGCC
CATCTGGGCGCGCCCGGATTACAACCCTCCACTGTTAGAGTCCTGGAAGGACCCGGACTACG
TCCCTCCGGTGGTGCACGGGTGCCCGTTGCCACCTATCAAGGCCCCTCCAATACCACCTCCA
CGGAGAAAGAGGACGGTTGTCCTAACAGAGTCCTCCGTGTCTTCTGCCTTAGCGGAGCTCGC
TACTAAGACCTTCGGCAGCTCCGAATCATCGGCCGTCGACAGCGGCACGGCGACCGCCCTTC
CTGACCAGGCCTCCGACGACGGTGACAAAGGATCCGACGTTGAGTCGTACTCCTCCATGCCC
CCCCTTGAGGGGGAACCGGGGGACCCCGATCTCAGTGACGGGTCTTGGTCTACCGTGAGCGA
GGAAGCTAGTGAGGATGTCGTCTGCTGC

Fig. 4B